Influence of Different Lignocellulose Sources on Endo-1,4-β-Glucanase Gene Expression and Enzymatic Activity of *Bacillus amyloliquefaciens* B31C

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Conversion of cellulose into fermentable sugars for ethanol production is currently performed by enzymatic hydrolysis catalyzed by cellulases. The cellulases are produced by a wide variety of microorganisms, playing a major role in the recycling of biomass. The endo-1,4-β-glucanase (CelB31C) from *Bacillus amyloliquefaciens* B31C, isolated from compost and previously selected on the basis of highest cellulase activity levels among *Bacillus* isolated, was characterized as being a potential candidate for a biocatalyst in lignocellulose conversion for second-generation bioethanol production. The aim of this work was to evaluate the changes in production of enzymatic activity of the endo-1,4-β-glucanase (CelB31C) and the expression of its gene (*bglC*) using a carboxymethylcellulase activity assay and qRT-PCR analysis, respectively, during growth of *B. amyloliquefaciens* B31C on different cellulose sources: carboxymethylcellulose (CMC), pure cellulose from *Arundo donax*, pretreated *Arundo donax* biomass (Chemtex), and microcrystalline cellulose (Avicel). The results showed that both the expression of *bglC* gene and the enzymatic activity production are related to the type of cellulose source. The strain showed a high enzymatic activity on lignocellulosic biomass and on microcrystalline cellulose. Furthermore, the highest gene expression occurred during the exponential phase of growth, except in the presence of Avicel.

Keywords: Bioethanol; Bacillus amyloliquefaciens; endo-1,4-β-glucanase; Arundo donax biomass; qRT-PCR; *bglC gene*

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INTRODUCTION

Lignocellulosic biomass is the most abundant renewable bioresource as a collectable, transportable, and storable chemical energy, and it is far from fully utilized. It is mainly composed of three major biopolymeric components: cellulose, hemicellulose, and lignin (Sathitsuksanoh *et al.* 2012). Strongly interwoven linkages among the biopolymers result in a naturally recalcitrant composite, and pretreatments are needed to make the cellulosic and hemicellulosic fractions accessible to enzymatic hydrolysis by opening the lignin sheath (Bhalla *et al.* 2013) and improving the enzymatic digestibility of pretreated lignocellulosic biomass.

In recent years, largely in response to an uncertain fuel supply and the need to reduce carbon dioxide emissions, bioethanol (along with biodiesel) has become one of the most promising biofuels today and is considered the only feasible alternative, in the

short and medium time frame, to fossil transport fuels in Europe and in the wider world (Onuki *et al.* 2008). To achieve energy and climate goals, the potential of bioenergy is a key issue (Ordóñez *et al*. 2013).

Bioethanol from traditional means, or first-generation bioethanol, is based on starch crops such as corn and wheat and on the bagasse byproducts from sugar crops such as sugar cane and sugar beet. Lignocellulose (excluding lignin) is an abundant carbohydrate source and has significant potential for conversion into liquid and gaseous biofuels (Bhalla *et al*. 2013).

In addition, the development of lignocellulosic technology has meant that not only high-energy content starch and sugar crops can be used, but also woody biomass or waste residues from forestry for 2nd generation biofuels.

The technology necessary to utilize the entire plants' biomass for ethanol production requires technologies that can break the cellulose into sugars and then ferment them to produce ethanol. Conversion of cellulose into fermentable sugars for ethanol production is currently performed by enzymatic hydrolysis catalyzed by cellulases, which are produced by a wide variety of microorganisms, depolymerizing raw materials and playing a major role in recycling of the biomass (Amore *et al*. 2012). Cellulases are needed in the hydrolysis step involved in second-generation ethanol for cellulose conversion into fermentable sugars, but costs for their production are still high; thus, efforts to improve the lignocellulose-to-ethanol conversion process are needed (Amore *et al*. 2013a). Despite its many advantages, cellulosic bioethanol is not yet industrially produced at a competitive level, mostly because of the high cost of cellulolytic enzymes. Because of this, more efficient and cheaper cellulolytic enzymes should be developed (Amore *et al*. 2013b).

Recently, 90 bacteria were isolated from raw composting materials obtained from vegetable processing industry wastes, using carboxymethylcellulose (CMC) as a carbon source (Ventorino *et al.* 2010; Pepe *et al*. 2013). A strain of *B. amyloliquefaciens* (B31C) was shown to produce the highest cellulase activity levels in comparison to the other isolates. The endo-1,4-β-glucanase CelB31C produced by *B. amyloliquefaciens* B31C was characterized as being a potential biocatalyst candidate in lignocellulose conversion for second-generation bioethanol production (Amore *et al*. 2013b).

The aim of this work was to evaluate the changes in cellulase activity and the expression of *bglC* during the three phases of growth of *B. amyloliquefaciens* B31C (lag, exponential, and early stationary) on different sources of cellulose. This is one of the most important factors affecting the production cost and yield of β-glucanase (Verma *et al.* 2013).

EXPERIMENTAL

Bacterial Strain, Media, and Growth Conditions

B. amyloliquefaciens strain B31C was used in this study; it was grown in liquid medium prepared as follows: 5 g L^{-1} CMC, 7 g L^{-1} yeast extract, 4 g L^{-1} KH_2PO_4, 4 g L^{-1} Na_2HPO_4, 0.2 g L^{-1} $MgSO_4.7H_2O$, 0.001 g L^{-1} $CaCl_2.2H_2O$, and 0.004 g L^{-1} $FeSO_4.7H_2O$ (Abou-Taleb *et al.* 2009). After an overnight incubation at 30 °C, a suitable volume of the broth culture was used to inoculate 30 mL of the same medium, modified in the cellulose composition. The different pretreated lignocellulosic biomasses and the commercial celluloses added in the liquid medium (10 g L^{-1}) are listed in Table 1.

Pretreated *A. donax* was received from Chemtex Italia S.r.l. One batch of steam-exploded material was used, with 1% (w/w) water-insoluble solids (WIS) (Table 1). During incubation at 30 °C, liquid culture samples, monitored by measuring the optical density (OD at 600 nm) and viable counting, were withdrawn during different stages of growth.

The experiments were performed in triplicates.

Table 1. Lignocellulosic Biomasses and the Commercial Celluloses Added

Source/Manufacturer	Commercial name - Origin
Sigma-Aldrich, Germany	Carboxymethylcellulose CMC Sodium Salt
Sigma-Aldrich, Germany	Avicel - Microcrystalline Cellulose
Arundo donax	Pure cellulose
Chemtex-Italia (GM-Group)	Pretreated *Arundo donax* Lignocellulosic Biomass

qRT-PCR Analysis

Total RNA was isolated from the microbial cells collected at different stages of growth, as described above, using a RiboPure™-Bacteria RNA isolation kit (Ambion, Milano, Italy), according to the manufacturer's instructions. Twenty nanograms of RNA (DNA-free) were first reverse transcribed in cDNA using iScript™ cDNA Synthesis; then, the gene of interest and the housekeeping gene (16S rRNA has been used as reference gene) were amplified using the iQ™ SYBR® Green Supermix Kit according to the manufacturer's instructions in a Chrom4 System Thermocycler (the kits used for the retrotranscription and the amplification, and the thermocycler, were purchased from Bio-Rad Milano). Based on the genome sequence of *B. amyloliquefaciens* FZB42 (GenBank: CP000560.1 - GeneID: 5461442), primers were designed to amplify portions of *bglC* codifying for the endo-1,4-beta-glucanase enzyme. All primers (Table 2) were purchased from Primm (Milano, Italy). The qRT-PCR running protocol was performed according to the manufacturer's instructions. To confirm that there was no background contamination, a negative control was included for each run. For each target gene, PCR efficiency was determined. Melt curves were calculated to check the amplified products.

Table 2. Primers Used for qRT-PCR

Genes	*Primers*	*T°m*	Efficency %	Fragment length (bp)	Source
16S-rRNA	f: CAAGCGTTGTCCGGAATTAT r: CTCAAGTTCCCCAGTTTCCA	60	101	112	This study
bglC	f: TAAGCTGGCTGAACGGCTAT r: TCCTGATCCGTTTCAGATCC	60	98	90	This study

The PCR efficiency (E) for each primer set was determined by generating cDNA dilution curves obtained by plotting the threshold cycle (Ct) for each cDNA amount against the log of the cDNA concentration.

The relative expression ratio was calculated for each gene of interest by using a mathematical model described by Pfaffl (2001) as follows:

$$\text{Ratio} = (E_{\text{target}})^{\Delta Ct,\ \text{target (control-sample)}} / (E_{\text{reference}})^{\Delta Ct,\ \text{reference (control-sample)}}$$

All measurements of gene expression were conducted in triplicates, and the mean of these values was used for the analysis.

Azo-CMCase Assay

The cells for the RNA extraction were collected by centrifugation at 12,000⊆g for 10 min, and the supernatants were collected to be processed for extracellular endo-1,4-ß-glucanase activity by AZO-CMCase assay (Megazyme, Ireland), following the supplier's instructions. The analytical determinations correspond to the mean value of three replicates.

RESULTS AND DISCUSSION

The growth of *B. amyloliquefaciens* B31C was monitored during lag, exponential, and early-stationary phases. No differences were found during the growth on the different cellulosic media used in this study (Table 1). Table 3 shows the number of cells (CFU mL^{-1}) and the OD value (600 nm) during the different stages of growth of the bacterium in different cellulose sources.

Table 3. Growth Phase Values of B31C

		CMC	Avicel	*A. donax*	Chemtex
Growth Phase	**OD 600 nm**	**CFU mL^{-1}**			
Lag (1.5 h) *	0.18	1.0×10^{6a}	3.0×10^{6a}	1.6×10^{6a}	1.5×10^{6a}
Exponential (3.5 h)	0.50	2.0×10^{7b}	4.0×10^{7b}	2.5×10^{7b}	2.0×10^{7b}
Sub-stationary (5.5 h)	0.80	2.0×10^{8c}	3.0×10^{8c}	2.8×10^{8c}	3.0×10^{8c}

* Time elapsed after inoculum. O.D. standard deviation < 0.002; CFU mL^{-1} <0.015;
The letters in the columns indicate significant differences $p \le 0.01$ (t-test).

The expression of *bglC* was determined by qRT-PCR as described earlier. Results, reported in Fig. 1, show an increase in *bglC* expression during the exponential phase of growth and a reduction during the early stationary phase. Regarding the carbon source, it was observed that an overexpression occurred during the lag phase, except in the presence of Chemtex's pretreated cellulose. During the following phases of growth, the degree of expression of the gene, compared to the 16S rRNA (reference gene), was detected 5.15-fold in the presence of Avicel, up to 300-fold in the presence of *A. donax*, rising generally to a level of expression higher than 10-fold during the early stationary phase.

The highest enzymatic activity (Fig. 2) was detected in the presence of Avicel, while the strain showed the lowest enzymatic activity in the presence of CMC. Generally, a slight variation of the activity during the different growth phases was noticeable. However, during the early stationary phase, the activity, except in the presence of Avicel, appeared higher. The pattern of enzyme production in the presence of different carbon sources during the early-stationary phase was Avicel > Chemtex pretreated biomass > *A. donax* > CMC.

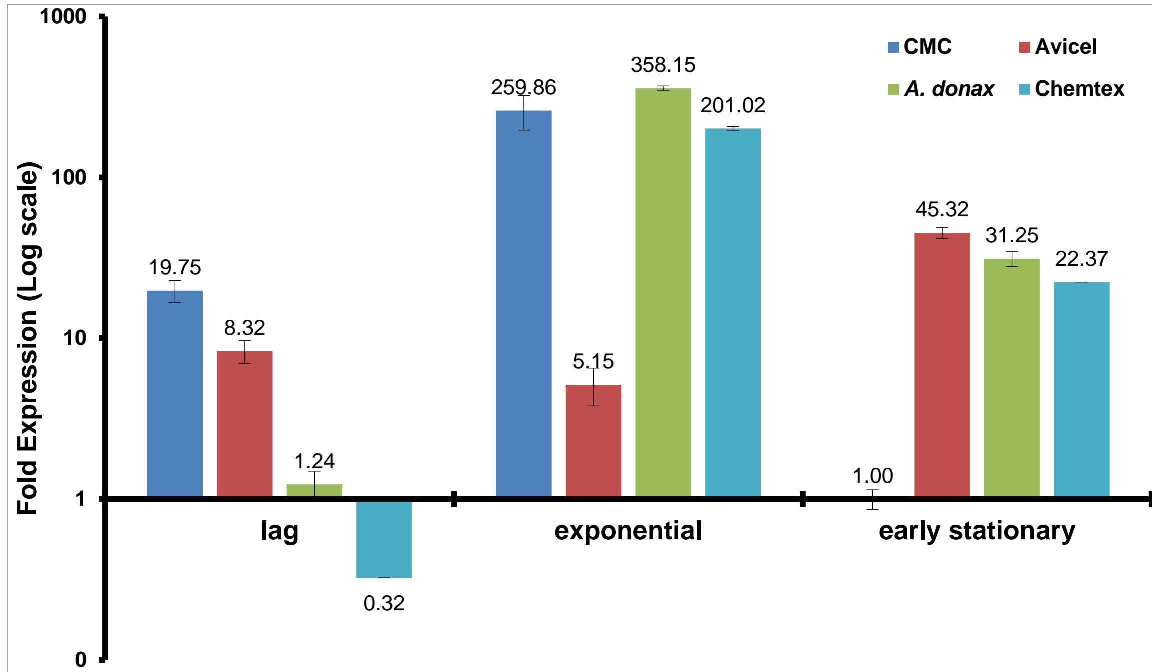

Fig. 1. Expression of *bglC* during different growth phases

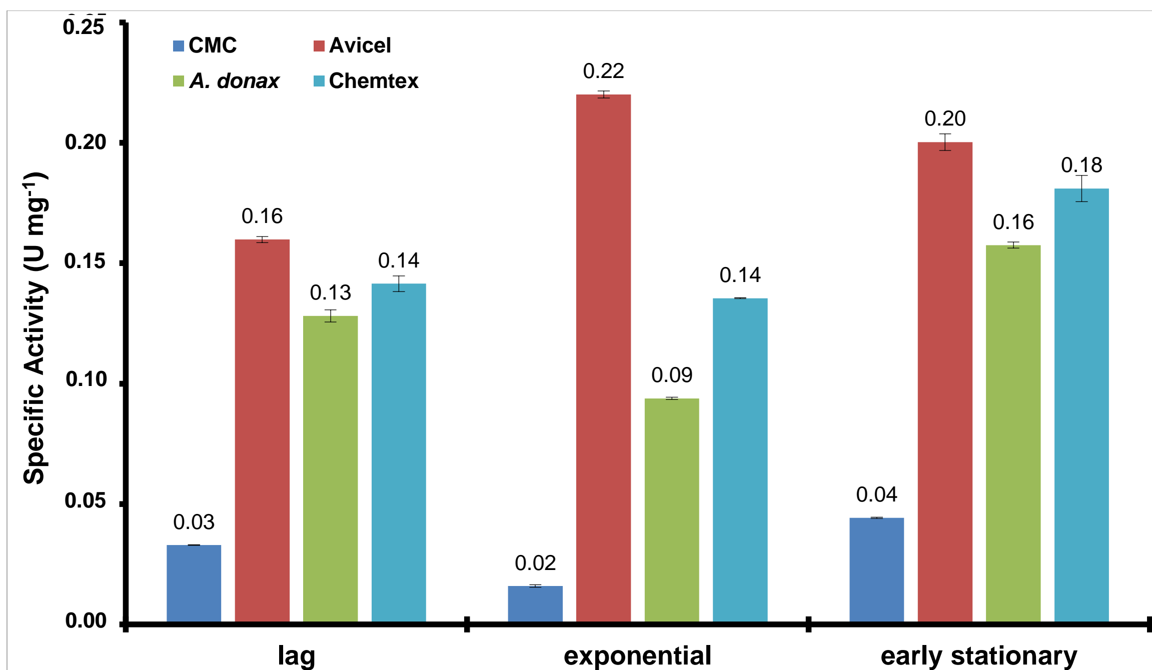

Fig. 2. Endo-glucanase activity with different cellulose sources (Azo-CMC assay)

In line with these results, Sethi *et al.* (2013) determined the effects of different agro-based waste source on the endo-cellulase activity of different bacteria isolated from soil, including a *Bacillus* strain. They underlined the involvement of the cellulose source on the enzymatic activity. As far as is known, this is the first report on the activity, as well as the gene expression, of the endo-1,4-β-glucanase of a *B. amyloliquefaciens* strain grown on different types of cellulose as carbon sources. A general overexpression of the investigated genes is evident from the results. On the other hand, this does not always

correspond to an increase of the enzymatic activity. It has been reported (Di Pasqua *et al.* 2013; De Filippis *et al.* 2013) that an increasing in gene expression does not always correspond to an increased protein regulation or high metabolites concentrations.

The gene expression increased in the presence of CMC more than in the presence of Avicel. This can be due to the microcrystalline structure of Avicel, the degradation of which requires a primary action of exo-cellulase enzymes (Soares *et al.* 2012). This could explain the lower expression of the gene compared to that in the presence of CMC during the lag and exponential growth phases. However, it is presumed that during the last phase of growth, in the presence of Avicel, the assumptive exo-cellulase enzymatic activity might had been replaced by endo-glucanase activity. This explains both the higher enzymatic activity and the high *bglC* expression. These findings are in line with those found by Wei *et al*. (2012). They reported that the expression pattern of cellulase activity takes place in a coordinated way that can enhance the overall efficiency of cellulose degradation.

An unexpected result was obtained in the presence of pure cellulose from *A. donax* and pretreated *A. donax* lignocellulose biomass because the abundance of lignin in the Chemtex biomass (data not shown) could reduce the enzymatic activity compared to that found in presence of pure cellulose from *A. donax*. A high induction of endo-1,4-β-glucanase, regardless of the concentration of lignin, has been reported by Bano *et al.* (2013). Recently, it has been proposed that lignin is melted and relocalized to the outer surface of the cell wall during high-temperature pretreatment, increasing the accessibility of the cellulose within, which might be a consequence of change in the S/G ratio of the lignin structure (S: syringyl-like lignin structures; G: guaiacyl-like lignin structures) (Li *et al*. 2010). S-rich lignin is more linear and often has a lower degree of polymerization.

It is tempting to speculate that the high-temperature pretreatment led to a relocalization and reorganization of the structure, increasing the S/G ratio, which in turn increased the enzymatic activity. Finally, the complex composition of *A. donax* lignocellulose biomass as multiple carbon source, could induce higher enzymatic activity (Xiong e*t al.* 2010).

CONCLUSIONS

1. This study showed that the enzymatic activity of *B. amyloliquefaciens* B31C strain is not related to the gene expression, representing a promising outcome for an application on a larger scale, to confirm the use of this enzyme as an interesting candidate for cellulose conversion in bioethanol production.
2. The growth of the *B. amyloliquefaciens* B31C strain was not affected by the different cellulose sources used in this study.
3. The pretreated lignocellulosic biomass (Chemtex) represents a good candidate for the industrial production of bioethanol.
4. Although the tests were done on a laboratory scale, the endo-glucanase activity of the *B.amyloliquefaciens* B31C strain was encouraging for defining the technical functions of the strain as promising for industry cellulose conversion in bioethanol.

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